cells also exhibited a non-desensitizing response to ACh.

On cells which had predominantly the fast desensitizing component (Type A, >90%), only ACh acted as a full agonist, whereas anatoxin A, nicotine, cytisine and the insecticide imidacloprid were all partial agonists. The agonist efficacy rank order was ACh ≫ anatoxin A ≫ imidacloprid = nicotine. Cytisine was the least effective agonist on these cells, eliciting a maximal response about 10 times lower than that observed for 1000 μM ACh. Anatoxin A was the most potent agonist, with an EC_{50} of 0.4 μM. On the same cell, ACh exhibited an EC_{50} of 13.5 μM. In contrast to imidacloprid ($EC_{50} \sim 1$ μM), nicotine had a relatively low potency, with an EC_{50} of 29 μM. These results gave a rank order of potency of anatoxin A > imidacloprid > ACh > nicotine for this component. On cells which had no fast-desensitizing component (Type B) ACh, anatoxin A, cytisine, nicotine and imidacloprid elicited nearly the same maximal responses at saturating concentrations. Anatoxin A was again the most potent agonist, with an $EC_{50} < 0.1$ μM. On the same cell, nicotine and cytisine exhibited practically the same EC_{50} of ~0.5 μM. ACh had the lowest potency with an EC_{50} of ~5 μM, and imidacloprid showed an EC_{50} of 3 μM. Taken together, these results gave a rank order of potency of anatoxin A > nicotine = cytisine > imidacloprid > ACh. With the exception of imidacloprid, the EC_{50} values for all agonists tested were lower than the corresponding EC_{50} values obtained from cells with mostly rapidly desensitizing ACh-induced currents. However, the most remarkable pharmacological difference between cells in which one or the other type of ACh response predominated is the clearly distinct affinity for nicotine, which can differ by a factor of 100.

In summary, our electrophysiological data confirm the existence of functional nAChR in locust neurones with different ion-channel properties and distinct affinity for ligands, suggesting that there exist (at least) two different and independent populations of nicotinic receptors in the insect nervous system. The same conclusions have been derived very recently by van den Beukel *et al*[9] when measuring the differential activation of nAChR subpopulations in *Locusta* neurones using physostigmine and acetylcholine.

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Chemistry, stereochemistry and biological properties of KWG 4168

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Abstract: KWG 4168 (8-*tert*-butyl-1,4-dioxaspiro[4,5]decan-2-ylmethyl(ethyl)(propyl)amine; proposed common name spiroxamine) is a new fungicide consisting of four biologically active isomers (two diastereomers, four enantiomers). The four isomers were separated by preparative HPLC on a chiral stationary phase. The diastereoisomers were synthesised from the corresponding chirally pure glycerol derivatives and were separated by preparative HPLC. COSY, HSQC and NOESY NMR spectroscopy were used to asign the configuration of the amino residue relative to the cyclohexyl ring. Studies of the activity against wheat powdery mildew, as well as the inhibition of sterol biosynthesis in fungi by the four stereoisomers, indicate the contribution of each isomer to the biological activity of spiroxamine.

Keywords: KWG 4168; spiroxamine; fungicide; biological activity; contribution of each isomer

1 INTRODUCTION

1,3-Dioxolan-4-methylamines, with the general structure **1** (Fig 1) have been synthesised as potential cereal fungicides within our studies on sterol biosynthesis inhibitors.[1] Coupling the residues R^1 and R^2 into a carbocyclic ring to produce spirocycles would be expected to reduce the number of the conformers, producing a better fit at the receptors in sterol biosynthesis. The spiroketalamine class of substances thus produced[2] has the general structure **2**. The first representatives of this class, 1-oxa- and 1,4-dioxa-

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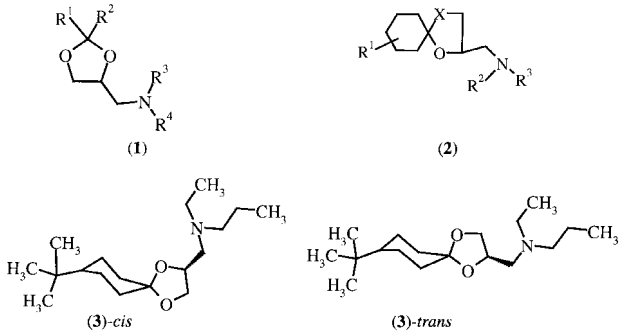

Figure 1. General structures of compounds discussed.

spiro-4,5-decane[3] showed good efficacy against cereal powdery mildew (caused by *Erysiphe graminis* DC) and acted as sterol biosynthesis inhibitors.

2 CHEMISTRY OF KWG 4168

Within the framework of an optimization programme of the spiroketalamines, 8-*tert*-butyl-1,4-dioxaspiro[4,5]decan-2-ylmethyl(ethyl)(propyl)amine (KWG 4168; proposed common name spiroxamine; **3**) has been synthesised using a very simple procedure (Fig 2; Scheme 1) in which *tert*-butyl cyclohexanone (**4**) is first reacted with racemic 3-chloro-1,2-propanediol. Ketal formation under acidic conditions leads to a *c*1:1 mixture of the diastereoisomers of 8-*tert*-butyl-1,4-dioxaspiro[4,5]decan-2-ylmethyl chloride (**5**) which is reacted with ethylpropylamine (**6**) to form the end product (**3**) as a result of nucleophilic substitution.

3 STEREOCHEMISTRY

KWG 4168 comprises *cis*- and *trans*-diastereoisomers (Fig 1) which can be separated by distillation of the corresponding chloroketals or by column chromatography; the A-form is coded KWG 4905 and the B-form KWG 5953. Separation of the enantiomers by HPLC is carried out on a Daicel-Chiracel-OD (10 μm) column (250 × 4.6 mm) at 40 °C using heptane + propanol (99.9 + 0.1, by volume) at a flow rate of 1 ml min^{-1}.[4]

4 SYNTHESIS OF THE *S*-ENANTIOMERS

Using the method of Tanaka *et al*[5] or of Baldwin *et al*,[6] the bis-ketal can be obtained by ketalisation of D-mannitol with *tert*-butyl cyclohexanone and it can then be cleaved to give the corresponding D-glycerol aldehyde by oxidation with sodium periodate. Subsequent reduction with sodium borohydride followed

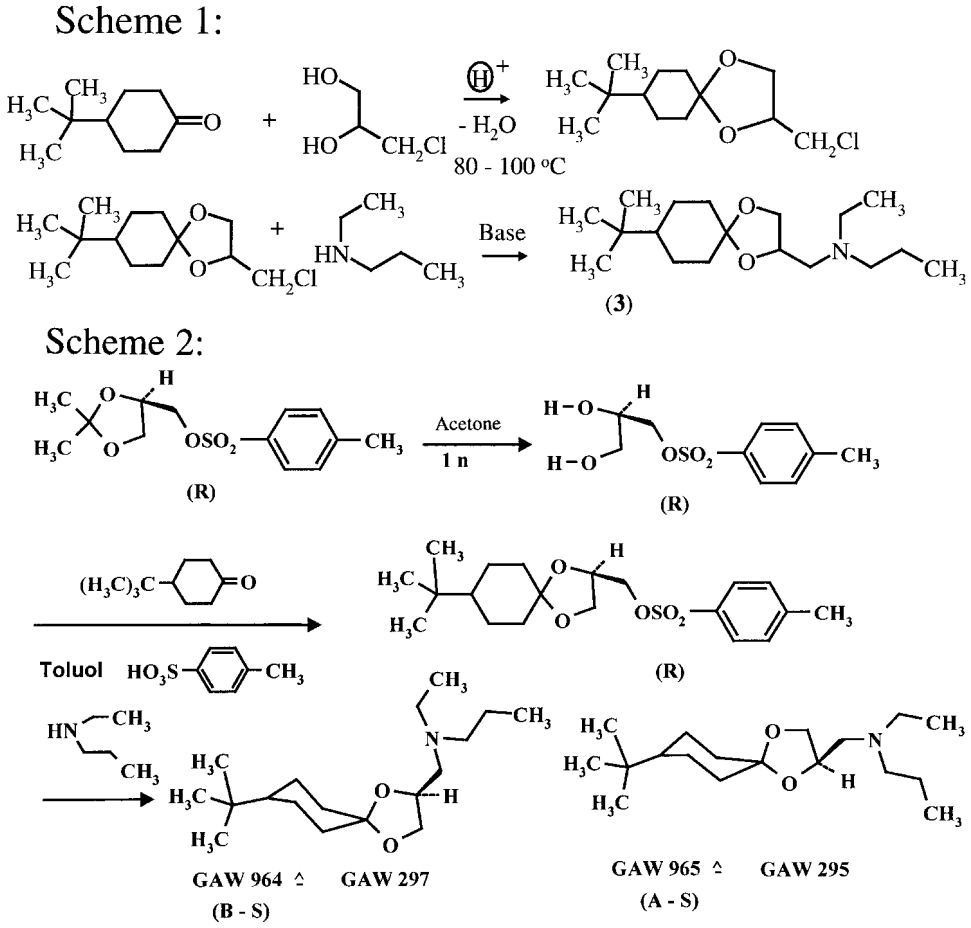

Figure 2. Synthetic routes to KWG-4168 and its isomers.

Table 1. Specific optical rotations of the four KWG-4168 isomers

Isomer	*HPLC Peak*	*Configuration*	*Optical rotation*[a]
GAW 295	3	eeS	+36.8
GAW 296	4	eeR	−38.4
GAW 297	1	eaS	+43.5
GAW 298	2	eaR	−38.4

[a] $[\alpha]_{20}$ (Hg 546) in heptane, c = 1.

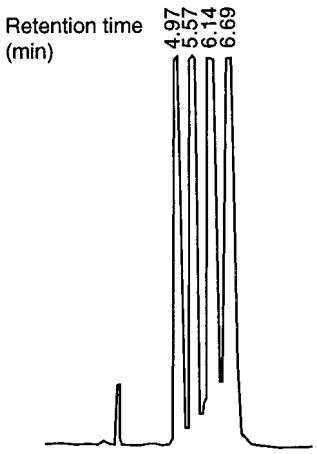

Figure 3. HPLC chromatogram of KWG-4168 on a chiral phase.

by esterification with methane sulfonyl chloride and reaction with *N*-ethylpropylamine leads to a diastereomeric mixture of the *S*-enantiomers which are coded GAW 297 and GAW 295.

4.1 Separation of the diastereoisomers to give the pure enantiomers

An alternative synthesis uses commercially available *R*-2,2-dimethyl-1,3-dioxolane-4-methanol and its corresponding *p*-toluenesulfonyl ester (Fig 2; Scheme 2). The *R*-configured isomers were obtained by an analogous procedure using *S*-2,2-dimethyl-1,3-dioxolane-4-methanol as starting material. The specific optical rotations for each isomer are given in Table 1.

Table 2. Spectrum of activity of KWG 4168 at 500–750g AI ha^{-1}

	Level of control		
Species	*Excellent*	*Good*	*Additional effects*
Wheat	*E graminis*	*P recondita*	*S tritici*
		P striiformis	*L nodorum*
Barley	*E graminis*	*P hordei*	
		P teres	
		R secalis	

5 NMR SPECROSCOPY

The mixtures of the enantiomers of peak 1 and peak 2 (ea*S*/ea*R*) and peaks 3 and 4 (ee*S*/ee*R*) (Fig 3) were used for the determination of the relative configuration of the amine residue [e and a denote the equatorial and axial orientation, respectively, of the *tert*-butyl group (first letter) and the substituted carbon of the cyclic acetal (second letter)].[4] By a full assignment of all proton signals using COSY-DQF[7] and GRASP-HSQC,[8–10] the assignment of the pseudoaxial and pseudoequatorial position of the amino residue was achieved by the observation of NOE peaks in the NOESY spectrum between proton 2 and proton 9ax in the ea*S*-isomer and between protons 3a and 3b and protons 7ax and 9ax, respectively in the ee*R*-isomer

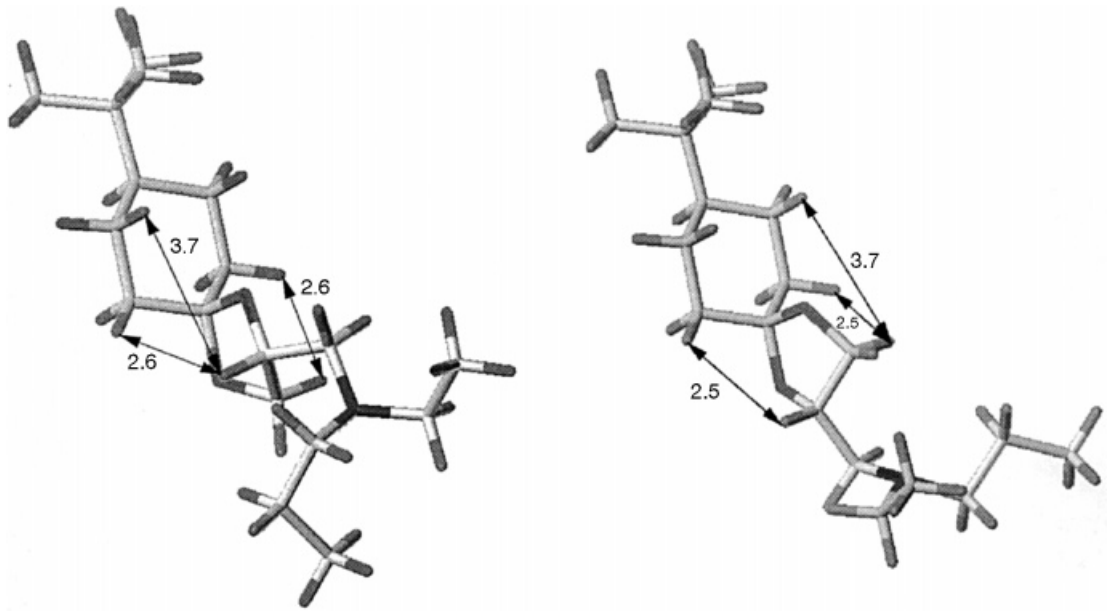

Figure 4. Conformations of the diastereomers GAW 964 and GAW 965 determined from NMR-NOE data and molecular modelling.

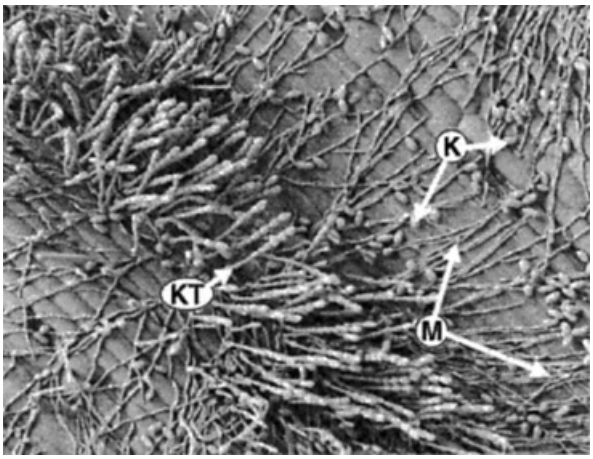

Figure 5. Untreated mildew colony 7 days after inoculation (KT = conidiophores, K = conidia, M = mycelium).

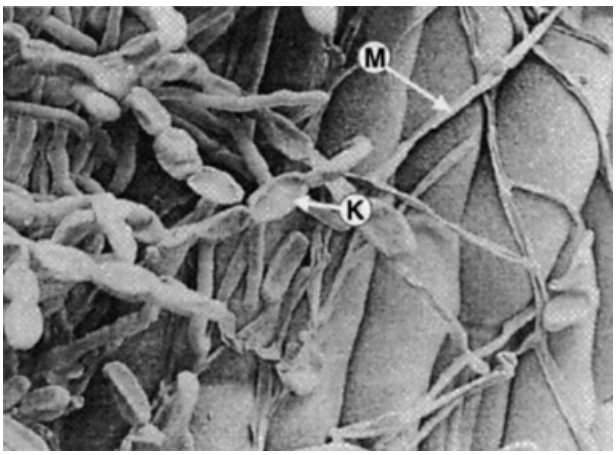

Figure 6. Mildew colony after exposure to KWG 4168 (100 μg g^{-1}) for 48 h.

(Fig 4). The stereoselective synthesis and the NMR spectroscopy data determined the structure of the enantiomers.

6 BIOLOGICAL ACTIVITY

KWG 4168 is an acropetally translocated systemic fungicide with excellent activity against powdery mildews (*E graminis* and *Uncinula necator* Burr). The activity spectrum also embraces other major diseases in cereals when used at 500–700 g AI ha^{-1} (Table 2).

Protective, curative and eradicative effects are shown against powdery mildews. The eradicative action, by which the product is able to stop an epidemic that is already under way, has been demonstrated convincingly in scanning electron microscopic studies (Figs 5 and 6). Figure 6 shows a seven-day-old mildew colony that was treated with KWG 4168 (100 μg g^{-1}) on the fifth day after inoculation; clearly, all the conidiophores and the individual conidia have collapsed. All four enantiomers are active against wheat and barley powdery mildew in greenhouse tests. KWG 4168 consists of the two diastereoisomers A [54 (+/− 2)%; KWG 4905] and B [46 (+/− 2)%; KWG 5953]. A gives rise to the two enantiomers GAW 295 (A/*S*) and GAW 296 (A/*R*) and B gives rise to the two enantiomers GAW 297 (B/*S*) and GAW 298 (B/*R*); activity data for the four enantiomers are given in Table 3.

7 BIOCHEMISTRY

The effect of KWG 4168 on inhibition of sterol biosynthesis has been investigated in comparison with that of morpholines. Autoradiography using labelled sterols indicated the presence of two bands in the lower sterol region similar to results with fenpropidin; only one band was visible with fenpropimorph. In a further study using various fungi and labelled sterol precursors (eg [^{14}C]acetate), the sterol pattern obtained with *Ustilago avenae* Rostr was very similar to that with barley powdery mildew, so that *U avenae* was used to determine the sterol biosynthetic activity of each enantiomer of spiroxamine; the results are in Table 4. From these it can be deduced that

	Protective/Curative activity			
Dose (mg litre^{-1})	*GAW 295 (A/S)*[a]	*GAW 296 (A/R)*	*GAW 297 (B/S)*	*GAW 298 (B/R)*
Erysiphe graminis f sp tritici				
100	88/100	88/100	50/75	79/58
25	88/100	84/100	63/25	75/25
5	50/50	41/25	25/0	25/0
2.5	50/25	41/0	25/0	25/0
1.0	0/0	0/0		
Erysiphe graminis f sp hordei				
100	100/100	100/100	84/88	88/88
25	100/100	100/100	84/25	88/25
5	50/88	50/25	50/0	25/0
2.5	50/25	50/0	25/0	0/0
1.0	0/25	0/0	0/0	0/0
0.5	0/25			

[a] See text for details.

Table 3. Biological activity of diastereoisomers and enantiomers of KWG 4168 against powdery mildews of wheat and barley in greenhouse tests

Table 4. Sterol distribution in *Ustilago avenue* after treatment with individual isomers of KWG 4168

	Sterol distribution in Ustilago avenae (%) isomer					
Sterol (%)	*Untreated*	*KWG 4168 (2.5 mg litre^{-1})*	*GAW 295 (A/S) (1.75 mg litre^{-1})*	*GAW 296 (A/R) (1 mg litre^{-1})*	*GAW 297 (B/S) (350 mg litre^{-1})*	*GAW 298 (B/R) (50 mg litre^{-1})*
$\Delta^{3,7-22}$-Ergostatrienol (ergosterol)	86.9	7.3	13.0	59.8	25.8	14.5
Δ^{5}-Ergostenol (campesterol)	–	–	–	–	14.3	1.8
Δ^{7}-Ergostenol (fungisterol)	7.1	2.1	4.5	4.4	6.3	4.3
Δ^{14}-Sterols:						
Δ^{8}14-Ergostadienol (ignosterol)	0.6	82.2	73.8	28.8	26.8	71.4
Δ^{8}-Sterols:						
Δ^{8}-Ergostenol	–	–	–	–	–	–
Δ^{8-22}-Ergostadienol	–	–	–	–	9.4	0.6
$\Delta^{5,8,22}$-Ergostatrienol (lichesterol)	–	–	–	–	8.9	–
Ergostatetraenol	–	2.4	3.0	3.6	–	–
Squalene	–	0.7	0.2	–	–	–
Unidentified sterols	5.0	5.2	5.5	3.4	8.6	7.3
Sterol content (μg/mg dry mass)	6.34	2.87	6.61	3.61	3.84	3.25
Growth (%)	100	92	50	30	11	39

Table 5. Summary of targets of isomers of KWG 4168

	Isomer			
Inhibition target	*GAW 295 (A/S)*	*GAW 296 (A/R)*	*GAW 297 (B/S)*	*GAW 298 (B/R)*
Squalene epoxidase	x			
Epoxisqualene cyclase		x		
Sterol Δ^{14}-reductase	x	x	x	x
Sterol $\Delta^{8\rightarrow7}$-isomerase			x	x

- spiroxamine inhibits sterol biosynthesis in fungi;
- the main target is Δ^{14}-reductase;
- the four enantiomers also inhibit squalene epoxidase, epoxysqualene cyclase and sterol $\Delta^{8} \rightarrow \Delta^{7}$ isomerase.

These observations are summarised in Table 5.

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